HISTONE METHYLTRANSFERASE IN HUMAN CANCER

A PROJECT REPORT SUBMITTED TO LIFE SCIENCES DEPARTMENT OF

NIT, Rourkela

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NATIONAL INSTITUTE OF TECHNOLOGY

ROURKELA, ODISHA

2011

DEPARTMENT OF LIFE SCIENCE
NATIONAL INSTITUTE OF TECHNOLOGY
ROURKELA-769008

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Date: 13.05.2011

CERTIFICATE

This is to certify that the thesis entitled "Histone Methyltransferase in Human Cancer" which is being submitted by Miss Monalisa Das, Roll No. 409LS2038, for the award of the degree of Master of Science from National Institute of Technology, Rourkela, is a record of bonafide research work, carried out by her under my supervision. The results embodied in this thesis are new and have not been submitted to any other university or institution for the award of any degree or diploma.

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ACKNOWLEDGEMENTS

This project is so far the most significant accomplishment in my academic life and it would have been impossible without people who supported me and believed in my caliber.

I would like to extend my gratitude and sincere thanks to my honorable supervisor **Prof. Samir Kumar Patra,** Associate Professor and Head of the Department of Life Science. I sincerely thankful for his exemplary guidance and encouragement. His trust and support inspired me in the most important moments of making right decisions and I am glad to work under his supervision.

I owe my sincere gratitude & thankfulness to other teachers; **Madam Bismita Nayak, Dr. Surajit Das** and **Dr Sujit Kumar Bhutia** of Department of Life Sciences, NIT Rourkela for showing sustained interest and providing help throughout the period of my work.

I express my special thanks to **Moonmoon Deb (**PhD Scholar**)**. No words to explain the help and care she showed towards me. I really learnt a lot from her.

I express my heartfelt thanks to other PhD scholars for their cooperation and help.

With my whole heart I deeply thanks to my friends **Minashree, Ria, Indu, Swayam,.Debashree ,Naina, Rasmi, Sushant, Surya & All**

Finally I dedicate whatever I achieve and attain to the gratefulness of my **parents** and the invisible hands of **almighty.**

Monalisa Das

DECLARATION

I, **Miss Monalisa Das**, M. Sc. Life Science, 4th semester, Department of Life Science, NIT, Rourkela hereby declare that my project work titled "Histone methyltransferase in human cancer" is original and no part of this work has been submitted for any other degree or diploma. All the given information is true to best of my knowledge.

Monalisa Das

Date: 13.05.2011

Place: ROURKELA

ABSTRACT

Histone lysine and arginine residues are related to post-translational modifications including methylation, phosphorylation, acetylation, ubiquitination, and sumoylation. The combination these modifications regulates critical DNA processes including replication, repair, and transcription. The size and function of eukaryotic genomes require that specific mechanisms for the stability of DNA. Such mechanism is the comprise of DNA with histones to form chromatin which helps in gene expression. The most important thins is that histones impact transcriptional activity is through site-specific enzymatic modification of the amino terminal histone “tails”, which can alter the spectrum of chromatin-associated proteins and hence transcriptional states. Among the well known modifications of histones, lysine methylation is the best one to represent a relatively stable mark which might be suitable for stable activation or repression, depending upon the site modified., The activity of enzymes that modify histone lysine and arginine residues have been correlated with a variety of human diseases like arthritis, cancer, heart disease, diabetes, and neurological disorders. Thus, this is important to understand the total kinetic and chemical mechanisms of these enzymes. Many HMTs have been linked to different types of cancer; however, in many cases we only have limited knowledge regarding the molecular mechanisms by which the HMTs contribute to disease development.

INTRODUCTION:

- Histones belong to a group of evolutionarily conserved proteins which play an important role in the proper packaging of DNA in the eukaryotic nucleus. DNA (~146 bp) together with histones (two each of histones H2A, H2B, H3 and H4) form the fundamental repeating subunit of chromatin & is known as nucleosome.
- It has been found that the human genome is composed of approximately three billion bases of DNA (3,000,000,000 bp), so we can infer that there are tens of millions of nucleosomes within a single human nucleus.
- Histones are in tight association with DNA, for which it has been postulated that they directly participate in many different DNA-templated programs including transcription, replication, recombination and DNA repair.
- But there are some confusion over the role of histones, for example if each tiny histone protein contains the same exact amino acid sequence as the other millions of histones in nucleus, how could they possibly direct distinct and opposing nuclear processes? Widespread research is being carried on worldwide to find a probable answer to this question and it has been found that histone code may be a possible answer to the above mentioned question.

HISTONE METHYLTRANSFERASE

- **Histone methyltransferases** (HMT) are group of enzymes consisting of histone-lysine N-methyltransferase and histone-arginine N-methyltransferase that catalyze the process of transfer of one to three methyl groups from the cofactor S-Adenosyl methionine to lysine and arginine residues of histone proteins. These protein is known to contain often a SET (**S**u(var)3-9, **E**nhancer of Zeste, **T**rithorax) domain. But recenly it has been discovered that HMT Dot1 lacks the characteristic SET domain.
- **Histone methylation** refers to the modification of certain amino acids in a histone protein by the addition of one, two, or three methyl groups. In the nucleus, DNA is wound around histones. Histones turns the genes in DNA "off" and "on", by methylation & demethylation respectively.

- This modification changes the properties of the nucleosome and affects its interactions with other proteins . Histone methylation is generally associated with transcriptional repression.

- Methylation of some lysine and arginine residues of histones causes transcriptional activation like methylation of lysine 4 of histone 3 (H3K4), and arginine (R) residues on H3 and H4.

- Histones mainly associates with DNA to form nucleosomes, which bundle to form chromatin fibers. They are globular proteins with a flexible N-terminus that protrudes from the nucleosome. The tail modifications are important characteristics of chromatin structure which plays an important role in regulation of gene expression that is for details of gene expression regulation by histone modifications.
- The nucleosome in eukaryotic organisms is the basic unit of chromosomes which composed of a double-stranded DNA wrapped around a protein octamer consisting of two copies each of the histone proteins H2A, H2B, H3, and H4.
- Histone proteins capable of various post-translational modifications including methylation, citrullination (deimination), acetylation, phosphorylation, ubiquitination, and simulations.
- The combining effect of these modifications results in important DNA regulatory processes including replication, repair, and transcription. Within histone proteins, lysine and arginine residues are abundant and highly post-translationally modified. **(Smith and Denu,2008)**
- Enzymes that modify these lysine and arginine residues have been correlated with ome of the human diseases such as rheumatoid arthritis, cancer, heart disease, diabetes and also with some neurodegenerative disorders such as Parkinson's disease and Alzheimer's disease. As a result of their involvement in a large variety of human disease states, it is highly important to understand the catalytic mechanisms of these enzymes. (Smith and Denu,2008)

Characterizing Histone Modification

- Histones are modified at a wide variety of sites.
- There are more than 60 different residues on histones where modifications have been detected by specific antibodies or by mass spectrometry.
- However, this represents lesser number of modifications that can take place on histones.
- Extra complexity arises partly from the fact that methylation at lysines or arginines may be one of three different forms: mono-, di-, or trimethyl for lysines and mono- or di- (asymmetric or symmetric) for arginines.
- This vast number of modifications has a great potential for functional responses, but it should be kept in the mind that all these modifications will not be on the same histone at the same time.
- The timing of the appearance of a modification is uncertain & will depend on the signaling conditions within the cell**.(Kouzaride Tonys,2007)**

Histone methyltransferases in cancer:

- The human genome contains almost more than 20,000 genes, but among these genes are expressed in each of the nearly 200 cell types found in an adult organism.
- Because of these differentiation of cells, certain genes are activated while others are repressed, which results in results in formation of a pattern of gene expression forming cellular identity.
- This pattern of gene expression is directed by transcription factors, which bind to specific promoter and enhancer sequences.
- Structure of chromatin plays an important role. Initially, chromatin was might be a static entity in which DNA is packaged to maintain the integrity of the molecule. Recently was found that the chromatin template undergoes dynamic changes during many cellular processes such as DNA replication and repair, recombination, cell cycle progression and transcription.

- The current view is that evolution of cancer is driven by the sequential obtaining of both genomic and epigenomic changes, consequently resulting in activation of oncogenes and the suppressor of tumor-suppressor genes .
- Epigenetic abnormalities in cancer comprise of many changes, including loss and gain of DNA methylation and alterations in histone methylation as well as acetylation.
- In contrast to genetic lesions, epigenetic alterations are potentially reversible, allowing for therapeutic intervention. The field of epigenetic therapy is rapidly expanding and inhibitors targeting histone deacetylases (HDACs) and DNA methyl transferases (DNMTs) have been approved for treatment of certain blood cancers and are being tested in clinical trials for different types of cancer. **(Albert and Helin,2009)**

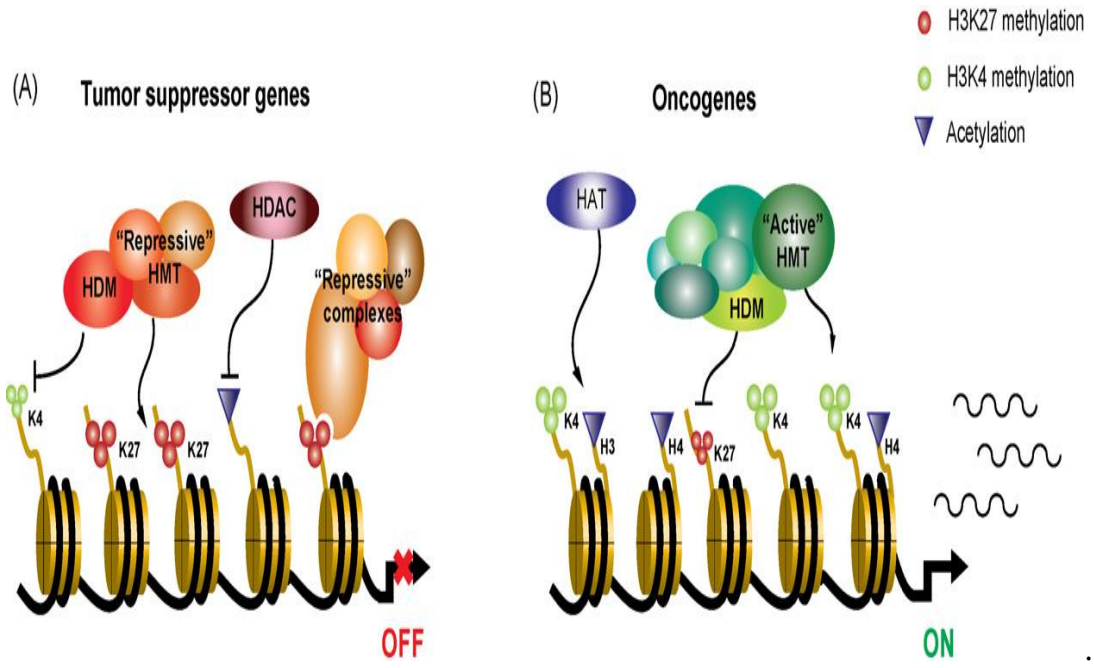


Fig-1 Misregulation of tumor-suppressors and oncogenes by histone methyltransferases. Both genetic and epigenetic mechanisms are thought to contribute to cancer development. While tumor-suppressor genes might be inappropriately silenced by repressive histone modifications (A), histone methylation and acetylation marks associated with active transcription might promote the expression of oncogenes, (B).HMT:histone methyltransferase;HDM:histone demethylase; HAT: histone acetyltransferase; HDAC**(Albert and Helin,2009)**

REVIEW OF LITERAURE OF G9a

ROLE OF G9a IN EHCC (Extrahepatic Cholangiocarcinoma) (Chen et al.,2007).

- Histone methyltransferase (HMTase) gene G9a composed of isonym protein i.e G9A, which transfers methyl groups from S-adenosyl-methionine to the lysine residues 9 and 27 of histone H3 (H3-K9 and H3-K27), methylation of histone H3-K9 is associated with the formation of chromatin, helps in gene expression, and regulation of DNA methylation in mammals .
- Recent studies showed that it is an important epigenetic mechanism of human malignant tumor. To investigate roles of G9a in tumor genesis and progression, they (Chen et al.,2007) detected the expression of G9a at the process of mRNA and protein and observed its clinical significance in extrahepatic cholangiocarcinoma (EHCC).
- As compared to other human histone methyltransferase such as SUV39H1 and ESET, it has been characterized to distinctive functions. Its first function is localization of G9a in nuclei suggested that this HMTase might target sites at transcriptionally active euchromatin rather than repressive percentric heterochromatin in which SUV39H1 family were localized .
- The second function is that G9a stronger HMTase activity to H3-K9 as compared to SUV39H1, and never be compensated for any other methyltransferases .
- The third function is that G9a transfers methyl groups to H3-K27 as well asH3-K9, whereas for SUV39H1 the targeting site was lonely Lys 9, recent study revealed that co-methylation of H3-K9 and H3-K27 is involved in gene silencing, specially for tumor suppressor gene.
- These function mentioned above decided G9a plays a key role in the regulation of, development, cellular differentiation and cell-cycle progression. The disruption and genetic loss of G9a led to disorder of H3-K9 and H3-K27 methylation may be resulted in tumorigenesis.. (Chen et al.,2007).

Downregulation of Histone H3 Lysine 9 Methyltransferase G9a Induces Centrosome Disruption and Chromosome Instability in Cancer Cells. (Kondo et al., 2008)

- Amino-terminal tails of Histone modifications affect access of regulatory factors and complexes to chromatin and this influence biological processes. Characteristics of cancer cell are prominent epigenetic dysregulation, including histone modifications. However, the functional roles of the histone methyltransferases (HMT) in cancer still remain unclear.
- Assembly of chromatin regulate a critical process related to, gene expression, progression through the cell cycle and DNA replicaton.
- Some modifications are associated with certain DNA template mediated processes
- One of the most well-studied histone modifications is methylation of histone H3 lysine 9 (H3K9) .After the initial identification of SUV39h1 in addition to SUV39h2 as a H3K9-specific histone methyltransferase (HMT) at least three other G9a, ESET/SETDB1 and EuHMTase1, HMTs, have been recognized for H3K9 in mammals .
- These enzymes have many affinities for the un-, mono- or dimethylated states and produce many methylation states. Studies in knockout mice for Suv39h1, h2 andG9a revealed that G9a is mainly responsible for (1Me) monomethylation and (2Me) dimethylation of H3K9, whereas Suv39h1 and Suv39h2 direct responsible for trimethylation (3Me) of H3K9.
- Furthermore, monomethylation and dimethylation on H3K9 primarily occured in euchromatin, while dimethylation and trimethylation on H3K9 were found within different types of heterochromatin, mainly at facultative and constitutive heterochromatin.
- The current study revealed that expression of SUV39H1 and G9a is important to enhance the growth of malignant cells and they play distinct roles in cancer cells. **(Kondo et al., 2008)**

Distinct Roles for Histone Methyltransferases G9a in Cancer Germ-Line Antigen Gene Regulation in Human Cancer Cells (Petra et al., 2009).

- Cancer germ-line (CG) antigens also called as cancer-testis antigens are targets of tumor vaccines currently undergoing world wide evaluation of clinical processes.
- Vaccine based on CG antigens in cancer includes both the inherent immunogenicity of these antigens and their restricted expression in normal human tissues as well as expression in human tumors .
- Principally regulated CG antigen genes at the transcriptional level by epigenetic signals.
- Lines of evidence are of following types:

(a) Hypomethylation of promoter DNA correlates with CG antigen gene expression in human tumor tissue and normal tissues .

(b) Treatment of non-expressing cancer cells with epigenetic modulatory drugs, including DNA methyltransferase (DNMT) and histone deacetylase (HDAC) inhibitors, plays an important role in CG antigen gene expression.

(c) Specific H3 modification patterns are associated with CG antigen gene expression level in human cancer cell.

- To facilitate the clinical development of CG antigen-targeted therapeutic approaches more detailed elucidation of the epigenetic mechanisms regulating CG antigen gene expression in human cancer cells and tumors should Disruption of DNMT enzymes in human cancer cells results in reduced H3K9me2 levels at CG antigen gene promoters,correlating with increased gene expression. .
- These data suggested G9a may play a primary role in CG antigen gene repression in human cancer cells. **(Petra et al., 2009)**

Hypoxic Stress Induces Dimethylated Histone H3 Lysine 9 through Histone Methyltransferase G9a in Mammalian Cells. (chen et al 2006).

- For chromatin organization and gene transcription an important mechanism are Post-translational modifications of histone amino-terminal tails, such as acetylation, methylation, ubiquitination, and phosphorylation, are important To date, histone acetylation and methylation are among the best-characterized modifications.
- Histone lysine acetylations are generally comprised with gene activation, whereas lysine methylation may have either positive or negative effects such as activation or silencing on transcription depending on the sites.
- Several H3K9-specific methyltransferases have been identified and characterized, as Suv39h1, Suv39h2, G9a, GLP EuHMTase1, and SETDB1/ESET. Among them, G9a and GLP EuHMTase1 are responsible for the dimethylation processes on H3K9 in vivo.
- The genetic alteration of either G9a or GLP/EuHMTase1 resulted in a loss of global H3K9me2 . G9a were found to be participated in gene silencing processes by interacting with many repressive transcriptional factors, such as, CDP/cut, PRDI-BF1/Blimp-1, NRSF/REST and SHP .
- Hypoxia is commonly present in many solid tumors and always occure when the growth of tumors outstrips their blood supply. In case of hypoxia, tumor cells increase the expression of genes related to angiogenesis, glucose and, glycolysis uptake using the transcription factor HIF .
- Hypoxia may also decreases the expression of several genes, such as cellular DNA repair proteins and adhesion proteins which leads to genetic instability and later which cause metastasis of tumor cells .
- H3K9-specific methyltransferase G9a is identified as one of the important cause of the hypoxia-induced H3K9me2.
- This conclusion is based on different lines of evidences, like hypoxia increased both G9a protein and HKMT activity as well as genetic alteration of G9a significantly induces the hypoxia-induced H3K9me2**.(chen et al 2006).**

OBJECTIVE

To check the expression of histone methyltranseferase in human cancer cell whether it is expressed or repressed. Our aim is to study the activity of G9a gene of histone methyltranseferase in human cancer.

"Comparative Analysis of **Histone Methyltransferase** (HMTase) **G9a** Expression in Normal and various Human Cancer Tissues".

MATERIALS AND METHODS

The Human blood was collected from CWS Hospital, Rourkela as normal human tissue and Gall bladder and Lymph node cancer tissues were collected from Calcutta Medical College, Kolkata.

a) Total RNA isolation

Reagents and Buffers:-

- TRIzol Reagents (Sigma),
- Choloroform,
- Isopropanol,
- Ethanol (70%),
- Denaturation Buffer
  - ✓ 50 % deionized formamide,
  - ✓ 2.2 M formaldehyde,
  - ✓ MOPS buffer (pH 7.0),
  - ✓ 6.6 % glycerol,
  - ✓ 0.5 % bromphenol,
- Ethidium Bromide (EtBr),
- Agarose

Protocol:-

- ✓ 50-100 mg of tissue in a 2 ml tube with 1 ml TRIzol was transferred.
- ✓ Homogenized for 60 sec in the polytron.
- ✓ 200 µl chloroform was added.
- ✓ It was mixed by inverting the tube for 15 sec.
- ✓ Incubated for 3 min at room temperature.
- ✓ Centrifuged at 12.000 g for 15 min.
- ✓ the aqueous phase was transferred into a fresh Eppendorf tube.
- ✓ 500 µl isopropanol was added.
- ✓ Centrifuged at max. 12.000 g for 10 min in the cold room.
- ✓ The pellet was washed with 500 µl 70 % ethanol.

- ✓ Centrifuged at max. 7.500 g for 5 min in the cold room.
- ✓ The pellet was dried on air for 10 min.
- ✓ Then the pellet was dissolved in 50-100 µl DEPC-H_2O.
- ✓ Incubated for 10 min at 60° C.
- ✓ Spectrophotometric reading was taken.
- ✓ Analysed the RNA on a MOPS gel:
    - ➢ 1-3 µg RNA was dissolved in 11 µl denaturation buffer.
    - ➢ 1 µl Ethidium bromide (1mg/ml) was added and denatured at 65° C for 15 min
    - ➢ 1 % agarose gel was loaded in MOPS buffer plus 5 % formaldehyde.
    - ➢ The gel was run at 40 V for 4 h.

b) cDNA synthesis (rt-PCR):

Regents and Buffer:-

- 5X First Strand Buffer
- 10mM dNTP Set
- 0.1M DTT
- Random Primers
- RNase OUT Ribonuclease Inhibitor
- Super Script II RNase H- Reverse Transcriptase

Protocol:-

- ✓ 8µl of total RNA were taken.
- ✓ Then 3 µl Random Primers was added.
- ✓ 1 µl dNTP mix was added.
- ✓ Then vortex and spin downed tube.
- ✓ Incubated at 65°C for 5 min.
- ✓ Placed tube on ice.
- ✓ 4 µl 5X Buffer, 2 µl DTT and 1µl RNAseOut were added.
- ✓ Then vortex and spin downed tube.

- ✓ Incubated at 42°C for 1 min.
- ✓ 1µl SuperScript II RNase H- Reverse Transcriptase was added.
- ✓ Incubated at 42°C for 60 min.
- ✓ Incubated at 70°C for 15 min.
- ✓ 180 µl of molecular grade water was added.
- ✓ Nano drop 1000 was used to measure concentration. Set sample typesetting to Other Sample and the constant to 33.
- ✓ Stored at -80°C.

c) Gene specific PCR:

Used Primers:-

Gene	Forward Primer	Reverse Primer
G9a	5'ATGGTGTGGGCTCCTCA-3'	5'TGAAGCTCAAGAGGTGA-3'
β actine	TCTACAATGAGCTGCGTGTG	TCCTGTCATCTCCTTCTGC

Chen et al (2007)

d) PCR mixture:- (Chen et al., 2007):

25 ml reaction volume

A typical reaction contained: template RNA 0.5 μg, up

down-stream primer 0.3 μL each,

10 × buffer 2.5 μL,

Mg2+(25 mol/L) 1.5 μL,

dNTP (2.5 mol/L) 2 μL,

Taq (5U/μL,Promega) 0.25 μL and

DEPC water 16.15 Ml

.

e) PCR conditions:- (Chen et al., 2007):

PCR reactions were performed in a PTC-200 thermal cycler (MJ Research, Watertown, MA)

95 ℃ for 2 min,

95℃ for 1 min,

58 ℃ for 1 min, and

72℃ for 1 min,

34 cycles,

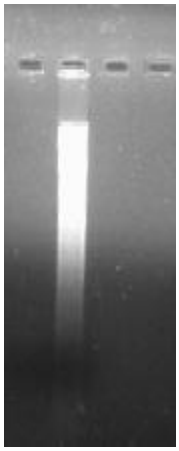
then 72℃ for 4 min.

RESULTS

RNA Extraction From Normal Tissue (Blood):

Product	**Conc^{n}.** (µg/ml)	**Purity**	
		260/280	**260/230**
Total RNA	570.32	1.34	0.82

Table.1: Spectrophotometer results of total RNA from blood tissue



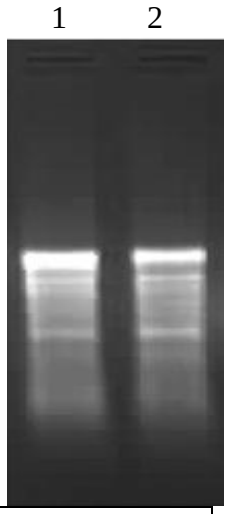
[Total RNA in 1% agarose gel]

[Total RNA in denaturation gel]

RNA Extraction From Cancerous Tissue:

Tissue	Conc^{n}. (µg/ml)	Purity	
		260/280	260/230
Gall Bladder Cancer	234.67	1.03	0.65
Lymph Node Cancer	478.51	1.61	1.02

Table.2: Spectrophotometer results of total RNA from cancerous tissue



Lane.1: Lymph Node Cancer

Lane.2: Gall Bladder Cancer

[Total RNA in denaturation gel]

Expression of G9a in Normal Blood:

Gene	Conc^{n}. (µg/ml)	Purity	
		260/280	260/230
G9a	280.69	1.50	0.76S

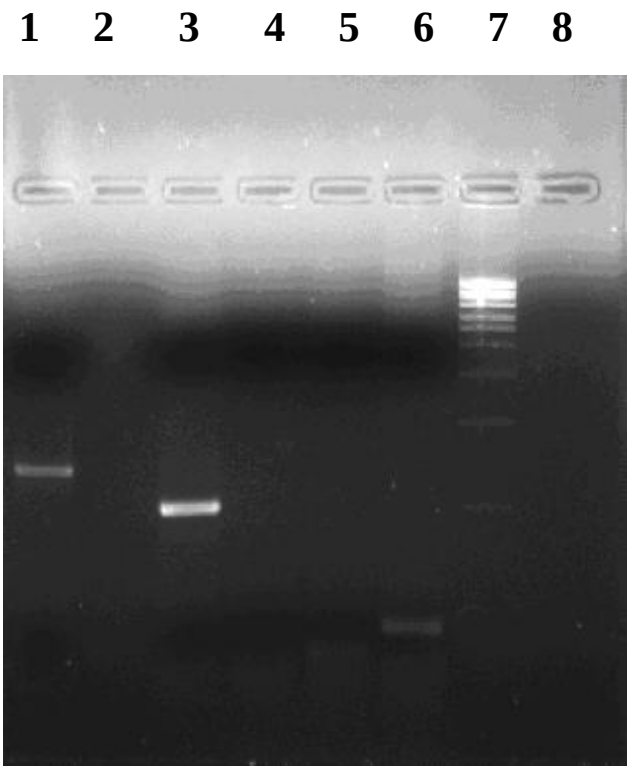
Table.3: Spectrophotometer results of gene specific amplification product from blood

G9a
[Gene specific PCR amplification]

Expression of G9a in Lymph Node Cancer

Gene	Conc^{n}. (µg/ml)	Purity	
		260/280	260/230
G9a	1820	0.98	1.27

Table.5: Spectrophotometer results of gene specific amplification product from lymph node cancer tissue



[Gene specific PCR amplification]

LANE 1:B actine

LANE 3:GADD45

LANE 4:G9a

LANE 7:DNA Ladder

DISCUSSION

After isolation the total RNA from normal blood and Cancerous tissue, we checked their concentration by taking its OD in spectrophotometer, in case of Gall Bladder the concentration was found to be very low i.e. 234.67µg/ml, which was very low compare to the other samples reading. Because it took a lot of time to processing after collecting the sample and also cDNA was not synthesize from the total RNA by rt-PCR method.

In case of lymph Node Cancer G9a is not expressed. This may be due to over production of other histone metyltransferases (for example, EZH2). Because of over expression of p300 lymph node cancer genome may be under the threat of drastic histone acetylation. Over expression of p300 may suppress H3K9 Methylation. EZH2 is overexpressed in case of lymph node cancer.

CONCLUSION

Studies of histone modifications and related molecular biology have entered an exciting phase, in which it has become possible to directly test the contribution of particular histone modifications to cellular and organism physiology.

Expression of G9a is present in many Cancer like Lung cancer, extrahepatic cholangiocarcinoma etc. But we find that repression of G9a in Lymph Node Cancer tissues.

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